Examining phenolic induction in *Fraxinus americana* in response to herbivory

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Abstract

Many plants contain chemical defenses known as secondary compounds; one of the most prevalent classes is known as phenolics. Certain plants have been known to induce phenolics in response to damage, while others maintain constant levels of phenolics even after damage. In order to determine if phenolic induction occurs in white ash trees (*Fraxinus americana*) in response to herbivory, leaflets were sampled to test for local induction (at the site of herbivory) and systemic induction (induction throughout the plant). Induction as a response to mechanical damage (*e.g.* slicing) was measured in order to remove a potential noisy variable from the results. Leaflets for the local/systemic test were eaten by forest tent caterpillars (*Malacosoma disstria*) and samples of induced leaflets were collected 24 hours after herbivory. The Folin-Denis analysis was used to determine the magnitude of change in phenolics between the constitutive and induced levels. Local induction was not found, but systemic induction was found to occur evenly between leaflets, despite predictions that there would be a spatial differences. One potential explanation for this was volatile gaseous compounds sending airborne cues to the undamaged leaflets, causing induction. Slicing was not found to cause significant levels of induction.

Introduction

Plants are under threat at every stage of their life cycle from both pathogens and herbivores. The damage caused by these pests (e.g. defoliation) can decrease the survivorship of plants by reducing their competitive ability (Levin 1976). In order to protect themselves from pathogens and herbivorous pests, plants have evolved a number of mechanical and chemical defenses. Plants have a mixture of waxes and lipids that act as a mechanical barrier. Trichomes serve as a defensive physical barrier against insects and many contain glandular defensive compounds such as resin (Swain 1977). Hard bark, thorns, and spines are also effective barriers against herbivorous pests. Mechanical barriers, however, are not the primary defense mechanism of plants. Chemical defenses, also referred to as "secondary compounds," are the primary determinants of whether insects eat or avoid plants. Insects have chemoreceptors on their mouths and antennae, which allow them to recognize even low levels of chemicals. These chemicals are often unpalatable as a way to indicate their toxicity, and they can cause adverse

physiological effects even at non-lethal levels. Without secondary compounds many plants would not be able to adequately defend themselves against the potentially devastating destruction wrought by insect pests (Swain 1977).

One of the most common and important classes of secondary compounds is called phenolics (Levin 1976). Phenolics act largely as deterrents by being unpalatable and, in mammal guts, binding with proteins that disrupt digestion. Phenolic compounds can inhibit healthy insect larval development and growth (Phillipe 2007). Most plants contain a constitutive level of phenolics, which are the baseline levels, but it may be an energy-intensive process to create and store the phenolics. There is a trade-off between phenolic production and growth and reproduction, so the constitutive levels are relatively low (Levin 1976). Additionally, if constitutive levels were always high, insect populations might evolve a resistance to the higher levels, thereby rendering this plant defense ineffective (Levin 1976). There are advantages for a plant to instead induce phenolics when it is under attack. Induction is an increase in the levels of defense (e.g. phenolics) usually in response to attack. This can allow resources to be used for growth and development when herbivory is not occurring (Phillipe 2007). Induction was demonstrated on quaking aspen (*Populus tremuloides*) by tearing the leaves to simulate damage done by third instar aspen tortrix larvae. The aspen showed short-term increased levels of phenolics, which was thought to be a mechanism to increase resistance to herbivory as the chemicals produced by the aspen were thought to reduce the food value of the leaves (Clausen, et al. 1989).

Chemical defenses can be induced either only locally (at the site of herbivory), or systemically throughout the plant (Phillipe 2007). It may be to a plant's advantage to "inform" distant parts of the plant when an attack is underway, so that defoliation is minimized. Plants send nutrients, water, and signal molecules through their xylem and phloem (Orians 2005, Farabee 2000). In the xylem, materials are transported acripetally, or toward the tip of the shoot (Sengbush 2003). There is evidence that signals molecules are involved in chemical induction, and these may be transported systemically through the xylem (though we could find no confirmation in the literature that this has been conclusively determined) (Orians 2005). The architecture of a leaf influences if and how signals are transported to other parts of the plant, including to other leaves. When a leaf is damaged, leaves that have direct vascular connections

[such as in tobacco (*Nicotiana*), cottonwood (*Populus trichocarpa*), and tomato (*Lycopersicon esculentum*)], will induce chemical defenses to a greater magnitude than in leaves that do not have direct vascular tissue connection (Orians 2005).

It is not known whether white ash (*Fraxinus americana*) induces phenolics when damaged. Other types of ash trees do induce when under herbivorous attack, including green ash (*Fraxinus pennsylvanica*), which induced phenolics when attacked by the emerald ash borer, a wood-boring insect (Chen and Poland, 2009). It is also not known how the architecture of the white ash leaf facilitates or constrains transfer of induction cues through the xylem. White ash leaves are compound, with leaflets located in symmetric pairs along the rachis (Figure 1). Xylem bundles may provide a constraint to chemical transportation, due to their acripetal nature, causing the materials to first be drawn to the shoot's tip before cycling through the remainder of the compound leaf. This would cause a delay in phenolic induction in leaflets that are on the opposite side of the rachis, as it would require the chemicals cross the xylem. Whether or not the leaf architecture presents a constraint is unknown, due to the limited knowledge of the vascular connections of the white ash leaf.

Figure 1: Compound Leaf Structure (Fraxinus americana)

In this study, we addressed the following questions:

1. Do white ash leaves induce when biotically damaged?
2. What is the spatial pattern of ash leaf induction within the compound leaf– is induction systemic or localized?
3. Does mechanical damage cause ash leaves to induce phenolics?

Materials and Methods

Study system

Our study was conducted at the University of Michigan Biological Station (UMBS) in the northern part of the Lower Peninsula near the town of Pellston. The property is almost completely designated as a nature research area, with minimal disturbances permitted. Our selected location was approximately 180 meters long along the beach in South Fishtail Bay. We

selected 10 white ash trees from this area that currently has a variety of tree species including *Quercus rubra, Pinus strobus, and Acer rubrum.* We sampled two leaves per tree, five leaflets total, on separate branches when possible, with one leaflet on one leaf used to determine the effect of mechanical damage on phenolic levels and the four others on the second leaf to determine the spatial pattern of induction. Only leaves with no to minimal visible mechanical or biological damage were used in our experiments.

Phenolic induction due to herbivory

To determine whether phenolic induction occurs due to herbivory, we used forest tent caterpillars (*Malacosoma disstria)* as our herbivores. We collected caterpillars based on approximate uniformity of size, in order to have caterpillars in a similar stage of life secreting similar salivary chemicals and eating approximately uniform amounts (Figure 2).

Figure 2: *Malacosoma disstria,* Courtesy of TrekNature

Figure 3: Caution taken to not damage midrib

In order to compare constitutive and post-damage phenolic levels in the leaflets, we first used a razor blade to cut alongside, but not into, the midrib from petiole to tip of leaflets (Figure 3). Damage to the midrib damages the xylem, and damage to the xylem would inhibit the flow of water and nutrients and cause an accumulation of nitrogenous compounds (Ohgushi 2007), and would likely have inhibited the leaflet's ability to transmit chemical signals to the rest of the leaf.

Spatial pattern of induction due to herbivory (localized and systemic)

To determine whether phenolic induction occurs due to herbivory, we removed 1/3 of leaflet 1 (Figure 4) as our constitutive sample, using a sharp razor blade and avoiding the

Figure 4: Leaflet 1 (after herbivory)

midrib. A sharp razor blade was used to minimize tearing of the leaf, with the hope that it would minimize induction due to mechanical damage. We removed 1/3 of the leaflet 1 because we needed to allow the caterpillars to consume as much of the leaf as possible while still leaving enough to quantify post-herbivory phenolics (we attempted to have the caterpillars eat 1/3 of the leaflet, and to leave 1/3 of the leaflet remaining after herbivory).

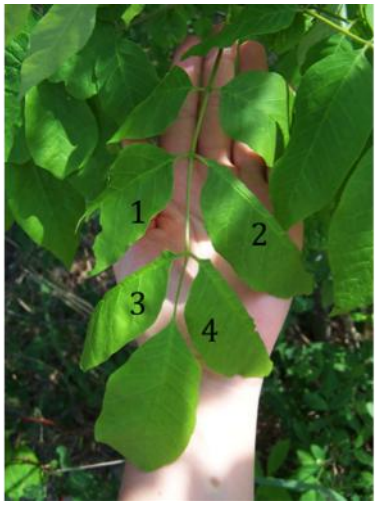


Figure 5: Leaf position numbering

After sampling leaflet 1, we removed ½ of the leaflets 2, 3, and 4 (Figure 5) and placed them in a glassine envelope, which was placed in a cooler filled with ice to slow any chemical reactions that may occur. To prevent subsequent herbivory, the leaf was isolated in a mesh bag.

Most of the caterpillars initially ate slowly, possibly due to the heat of the day. In order to keep the caterpillars on leaflet 1, we put the caterpillars in Ziploc bags we had altered and then sealed the caterpillars on leaflet 1. We tore holes in the Ziploc bag with a needle, in order to prevent suffocation of the caterpillars, and then taped the bags to make them smaller in order to keep the caterpillars on the leaf as much as possible (Figure 6). We checked the bags regularly to prevent over-consumption. When approximately 1/3 of the leaflet had been consumed, we removed the caterpillars. We returned to each tree approximately 24 hours later and removed the remaining half of the leaflet. We chose to wait 24 hours because there is evidence that phenolic levels peak between 24 and 72 hours after damage, and then fade after the attack ceases (Baldwin 1989, Clausen et al. 1989). Because we did not have to worry about causing induction when we were taking our post-herbivory sample, we used scissors and were sure not to include the midrib in our post-induction sample, as the presence of the midrib would alter the phenolic levels of the sample.

Figure 6: Caterpillar in Ziploc bag

When we had collected samples from 10 trees, we placed our samples in an -80° F freezer until we could dry them in a lyophilizer. When our samples were dry, we kept them in a

dessicator to prevent water from condensing onto the leaf powder. We ground up our leaflets in order to maximize available surface area for chemical reactions, and we used either a mechanical grinder or a mortar and pestle, depending on what was available to us at the time and depending on the sample mass (the mortar and pestle was used on very small samples, in order to minimize sample loss). We added liquid nitrogen to the leaves before grinding in order to make them more brittle in order to produce a finer leaf powder. When the samples were ground, we used the Folin-Denis analysis to determine the relative concentration of total phenolics (see Appendix).

Effect of mechanical damage on phenolic induction

In order to determine if the act of cutting leaves with a razor blade during collection was acting as a noisy variable in our spatial induction test, we conducted a separate test to determine whether mechanical damage caused phenolic induction in ash leaflets and, if so, to what degree. We were unable to find literature indicating whether white ash trees induce phenolics when subjected to mechanical damage. If mechanical damage caused induction, we would have to correct our estimates of herbivore-induced changes in phenolic levels. We would do this by subtracting the relative change in percent dry weight of phenolics produced by the mechanical damage from the relative change in percent dry weight of phenolics from the herbivorous experiment.

Our sampling procedures were identical to those described above except that only one leaflet was used, because we were not measuring the effect of damage over distance but just if a response does occur. The cut leaflets, off of ten trees, were placed in a glassine envelope, which was kept in an ice-filled cooler, and the branch was isolated using a mesh bag to prevent further herbivory. Twenty-four hours after the leaflet's initial exposure to the herbivory, we used scissors to remove the second half of the leaflet and prepared them in the same manner as described above. We ran the Folin-Denis analysis on these samples as well (see Appendix).

Statistical Analyses

Paired design

We used a paired t-test because we are interested in comparing the constitutive levels of phenolics to the induced levels of phenolics in the same leaflet. We compared the absolute

change of phenolics, which is the increase in the percent dry weight phenolics in each leaflet. This will determine if localized or systemic induction occurs. Prior to running the paired t-test, normality was confirmed.

We also did a separate paired t-test to compare the magnitude of phenolic induction between leaflet positions in order to determine if any position induced more than any others. This will help us determine if spatial distance between leaflets affects systemic induction. Normality of the distribution of the data was confirmed prior to running the test.

One-sample t-test

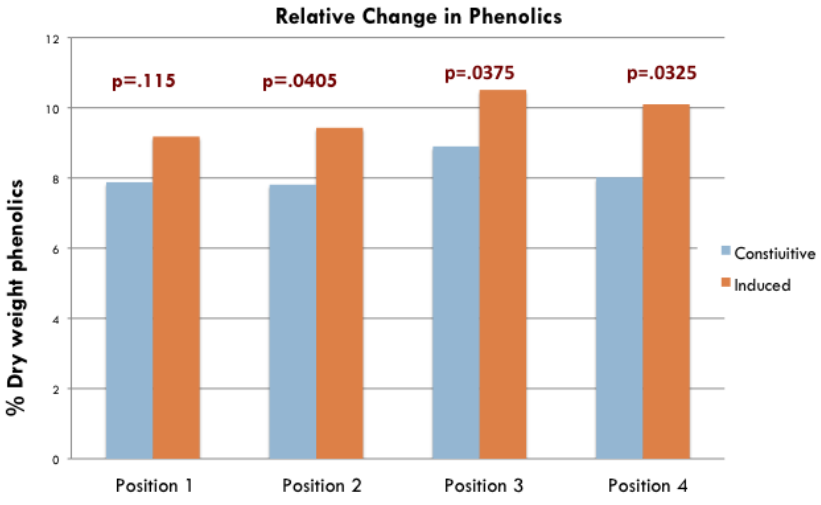
Another test we ran was a one-sample t-test after calculating the difference between constitutive phenolic levels and induced phenolic levels for each leaflet. This is called the relative change in percent dry weight of phenolics. We used a one-sample t-test to ask whether on average the percent dry weight change in phenolic levels was greater than 0.

Results

Phenolic induction due to herbivory

Paired design

Damage by forest tent caterpillars did not cause local induction of phenolics. Constitutive levels in leaflet 1 averaged 7.8 ± 2.0 percent leaf dry weight of phenolics and post-damage levels averaged 9.2 ± 3.5 percent leaf dry weight of phenolics, yielding an increase of 1.3 ± 3.7 percent leaf dry weight; this difference was not statistically significant (t=-1.1, d.f.=9, p=0.15). Constitutive levels in leaflet 2 averaged 7.81 ± 2.22 percent leaf dry weight of phenolics and post-damage levels averaged 9.4 ± 2.6 percent



dry weight of phenolics, yielding an increase of 1.6 ± 2.7 percent leaf dry weight; this difference was not statistically significant (t=-1.9, d.f.=9, p=0.046).

Constitutive levels in leaflet 3 averaged 8.9 ± 3.0 percent leaf dry weight of phenolics and post-damage levels averaged 10.5 ± 3.1 percent dry weight of phenolics, yielding an increase of 1.6 ± 3.2 percent leaf dry weight; this difference was not statistically significant (t=-1.6, d.f.=9, p=0.075).

Constitutive levels in leaflet 4 averaged 8.0 ± 1.6 percent leaf dry weight of phenolics and post-damage levels averaged 10.1 ± 3.6 percent dry weight of phenolics, yielding an increase of 2.1 ± 3.5 percent leaf dry weight; this difference was not statistically significant (t=-1.9, d.f.=9, p=0.046).

Damage by forest tent caterpillars did cause significant increase of phenolics in leaflets 2 and 4, and nearly significant increase of phenolics in leaflet 3 (t=-1.9, -1.6, -1.9, d.f.=9, p=0.046, 0.075, 0.046). Because it was unclear whether leaflet 3 did or did not induce, the one-sample t-test was used to see if we could get more powerful results.

One-sample t-test

At position 2 there was an increase in relative change (percentage change in percent dry weight of phenolics) of 27.2 ± 43.8. Position 3 had a relative change increase of 28.6 ± 44.9. Position 4 had a relative change increase of 28.9 ± 43.7. It appears that damage by forest tent caterpillars did cause induction in leaflets 2, 3, and 4 (t= 1.97, 2.0, 2.1, d.f=9, p=0.04, 0.04, 0.033). At position 1 there was an increase in relative change of 27.2 ± 43.8. Damage by forest

Leaflet Position	Mean Constitutive	Mean Induced	Mean Absolute Change	p-value	Mean Relative Change	p-value
1	7.9 ± 2.0	9.2 ± 3.5	1.3 ± 3.7	0.15	19.8 ± 47.8	0.11
2	7.8 ± 2.2	9.4 ± 2.6	1.6 ± 2.7	0.046	27.2 ± 43.8	0.04
3	8.9 ± 3.0	10.5 ± 3.1	1.6 ± 3.2	0.075	28.6 ± 44.9	0.04
4	8.0 ± 1.6	10.1 ± 3.6	2.1 ± 3.5	0.046	28.9 ± 43.7	0.033

tent caterpillars did not cause induction in leaflet 1 (t=1.308, d.f.=9 , p= 0.11).

Effects of spatial arrangement of leaf on systemic induction

It was determined from the one-sample t-test that damage due to forest tent caterpillars did occur in leaflets 2, 3, and 4, therefore systemic induction was confirmed. We compared leaflets 2 and 4, leaflets 2 and 3, and leaflets 3 and 4, in three separate paired t-tests (t=-0.8, -0.1, -0.03, d.f.=9, p=0.9, 0.9, 0.9). We did not include leaflet 1, because induction did not occur. There was no significant difference in the levels of phenolics between leaflets of different positions. The results showed that we could not determine if the position of the leaflets have an effect on the magnitude of induction.

Discussion

The results of our research indicated that induction does occur in white ash. Based on our first experiment, localized induction did not occur in leaflet 1, but induction did occur in the remaining leaflets 2, 3, and 4. From this we concluded that systemic induction did take place throughout the leaf; however, since the overall increase in dry weight phenolics was not different among leaflets, the spatial arrangement of the leaflet did not play role in induction. As a final test, phenolic induction due to mechanical damage was measured and our results suggest that it had no effect on the increase in phenolics.

Mechanical damage presented by the razor blade did not cause a significant increase in phenolics. The use of the razor blade was chosen based on the fact that other methods of mechanical damage (e.g. crushing leaf tissue) have been known to cause phenolic induction (Korth & Dixon 1997). However, damage due to herbivory causes a higher increase in the level of phenolics than mechanical damage (Korth & Dixon 1997). This was considered to be due to the fact that saliva from caterpillars will activate elicitors, which initiates the production of phenolics (McNeil 2010). The razor blade lacked the bacteria present found in saliva, and did not cause a significant increase in phenolic levels.

Our study indicates that induction did not occur in leaflet 1, the leaflet that was eaten by the caterpillars, but occurred in three other leaflets that were opposite and/or distal to the

damaged leaflet. This was surprising since we expected that if induction does occur in some if not all the leaflets, we expected leaflet 1 would induce because it was the site of herbivory. When caterpillars eat certain plants, the bacteria present in the saliva activate elicitors that stimulate the octadenoid and jasmonate biosynthesis cascade. In the jasmonate pathway, jasmonic acid is responsible for initiating up-regulation of genes which onset protein transcription (McNeil 2010). After proteins are transcribed, enzymes involved in synthesis of phenolics can be made. In addition, since leaflet 1 had only a small fraction of the original leaflet remaining, the ash tree may have sent induction cues to other areas of the plant where protection from herbivory was worthwhile (i.e. leaves 2, 3 and 4) rather than invest nutrients in leaflets with lower fitness. Nitrogen is used in producing enzymes that catalyze synthesis of phenolics, and it seems probable that natural selection would favor allocating nitrogen to undamaged leaflets, to better protect them and increase survivorship. One possible reason that leaflet 1 did not show increased amounts of phenolics could be due to the allocation of metabolic resources (e.g. nitrogen) used for growth. The ecological costs of phenolics could affect the fitness of the plant due to the trade-offs between using resources for defense and using resources for growth and reproduction.

Another possible explanation for why induction was not seen in leaflet 1 could be caused by the decrease in photosynthesis. Studies of wild parsnip (*Pastinaca sativa*) have shown that as the induction of phenolics increased, a decrease in photosynthesis and increase in respiration occurred (Zanger et al. 1997). This would lead to a decrease in the amount of carbon available to the leaflet, which is required for synthesis of phenolics. Leaflet 1 was the most damaged of the four leaflets sampled and as a result may have allocated its resources for photosynthesis (growth) instead of phenolics (defense).

It is also probable that resources such as nitrogen were moved out of the leaflet to surrounding leaflets, which resulted in the lack of induction. The levels of defense and photosynthesis may not always be inversely related, sometimes both defense and growth can be affected negatively. There are instances when it may be more advantageous to protect the leaves that are the most productive (Tang et al. 2009). Phenolics are derived from the intermediates of photosynthesis (e.g. PGA and PEP), so as photosynthesis decreases so do phenolics. Since leaflet 1 had been damaged by the herbivores, it had the least amount of biomass and thus fewer intact chloroplasts for photosynthesis. This would decrease the amount of photosynthesis,

causing an indirect inverse affect on the levels of phenolics (Zangeral and Berenbaum 1998). Overall, there could be many adverse side effects that a decrease in photosynthesis has on the levels of phenolics.

When we first determined that leaflet 1 position did not show a significant increase in phenolic levels, we thought it could be due to some of the samples having small dry weights (e.g. induced leaflets from trees D, K, & J). Due to the extent of caterpillars feeding, the collected sample amounts for each of these were 10.9, 5.1, and 6.6 mg., respectively. The relative change of percent dry weight of phenolics, was 8, 122.9, and -25.6 % respectively. Two out of the three small samples indicated an increase in phenolic levels. Therefore, we cannot suggest that small amounts of leaf powder led to the lack of significance of the statistical test for leaflet position 1.

When we compared the phenolic levels between the different leaflet positions (not including leaflet 1), our results indicated the increase in percent dry weight of phenolics were not significant different. We concluded that leaflets 2, 3, and 4 induced equally, despite expecting that there would be differences in phenolic levels due to spatial position of leaflets. In particular we expected that the phenolic levels would decrease in intensity with distance from the damaged leaflet. One possible reason for the comparable levels of induction could be that the xylem transports cues in ways different from how we expected. The xylem may not act as a barrier to the other half of the leaf, leading to rapid spread of induction. Additionally, the cues could travel down the xylem towards the shoot, and then back down the other side of the leaf. By only looking at the phenolic content based on 24-hour intervals, we could have missed cues spreading more quickly than we were able to detect.

It is additionally possible that cues do not only travel through the xylem, but may travel by other means. There is evidence that cues may be sent via volatile compounds released into the air when damage occurs. Volatile compounds can signal to undamaged parts of the plant (and even to other plants) that danger is on the way (Dicke et al. 2003). It is possible that leaflets 2, 3, and 4 (which did not experience herbivory) received cues from the air that activated their defenses. It would be interesting to further investigate whether volatile compounds release signals and if so, whether they are evenly distributed throughout compound leaves.

In the event of further experimentation, there are other issues that may want to be included in the experimental design. Some points of interest are: measuring the amount of nitrogen within the leaflets, measuring levels of photorespiration, and increasing the sample size. Nitrogen is a limiting resource in plants. If other researchers were able to obtain sufficient amounts of nitrogen samples, they would be able to see if the induced leaflets had different amounts of nitrogen and would be able to eliminate it as a potential noisy variable. Also, we learned that increasing carbon dioxide levels decrease the amount of phenolics induced. A future study could measure how the phenolic levels differed in relation to the amount of carbon dioxide released. Lastly, an increased sample size would increase confidence in the results of the experiment.

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